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## **Lentiviral gene therapy vector with UCOE stably restores function in iPSC-derived neutrophils of a CDG patient**

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# Lentiviral gene therapy vector with UCOE stably restores function in iPSC-derived neutrophils of a CDG patient

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## Abstract

A recent gamma-retroviral clinical Chronic Granulomatous Disease (CGD) gene therapy (GT) trial achieved proof-of-concept but was accompanied by activation of oncogenes and transgene silencing. The *ubiquitous chromatin opening element* (UCOE) comprises the sequences of two divergently oriented house-keeping gene promoters and is known to have anti-silencing properties. In a screen we identified two novel UCOE constructs that prevent adjacent promoter methylation in P19 cells. Experiments were continued with the shorter UCOE constructs in induced pluripotent stem cells (iPSC) derived from a p47phox-deficient CGD patient. The iPSC line was transduced with the lentiviral GT vectors expressing p47 under the constitutively active SFFV promoter with UCOE element (UCOE\_SF) and without UCOE element (SF) adjacent to the SFFV promoter. The iPSC were expanded before propagation towards neutrophils. 20 days after transduction the UCOE\_SF vector was protected from methylation in iPSC as previously shown in P19 cells, whereas the SF vector was heavily methylated in iPSC. The UCOE\_SF vector maintained stable transgene expression in iPSC, macrophages and neutrophils, whereas the SF vector was strongly silenced. The UCOE\_SF vector stably restored ROS production in neutrophils, whereas for the SF vector the count of ROS producing cells was marginal. To conclude, we have shown that the prevention of transgene silencing by UCOE is functionally and mechanistically preserved upon terminal neutrophil differentiation.

## Introduction

The primary immunodeficiency Chronic Granulomatous Disease (CGD) is caused by a defective NADPH-oxidase complex, which is the molecular machinery required for the production of reactive oxygen species (ROS) that facilitate microbial killing by phagocytes. The disease is characterized by recurrent severe bacterial and fungal infections requiring prophylactic treatment of the patients with antibiotics and antimycotics. The state-of-the-art curative therapy for CGD is bone marrow transplantation if a HLA identical donor is available. Unfortunately, depending on the ethnic group, a HLA identical donor is available for only about 16–75% of CGD patients [1]. For patients without HLA matching BM donor, a gene therapy (GT) approach using autologous bone marrow cells might be an alternative therapeutic option.

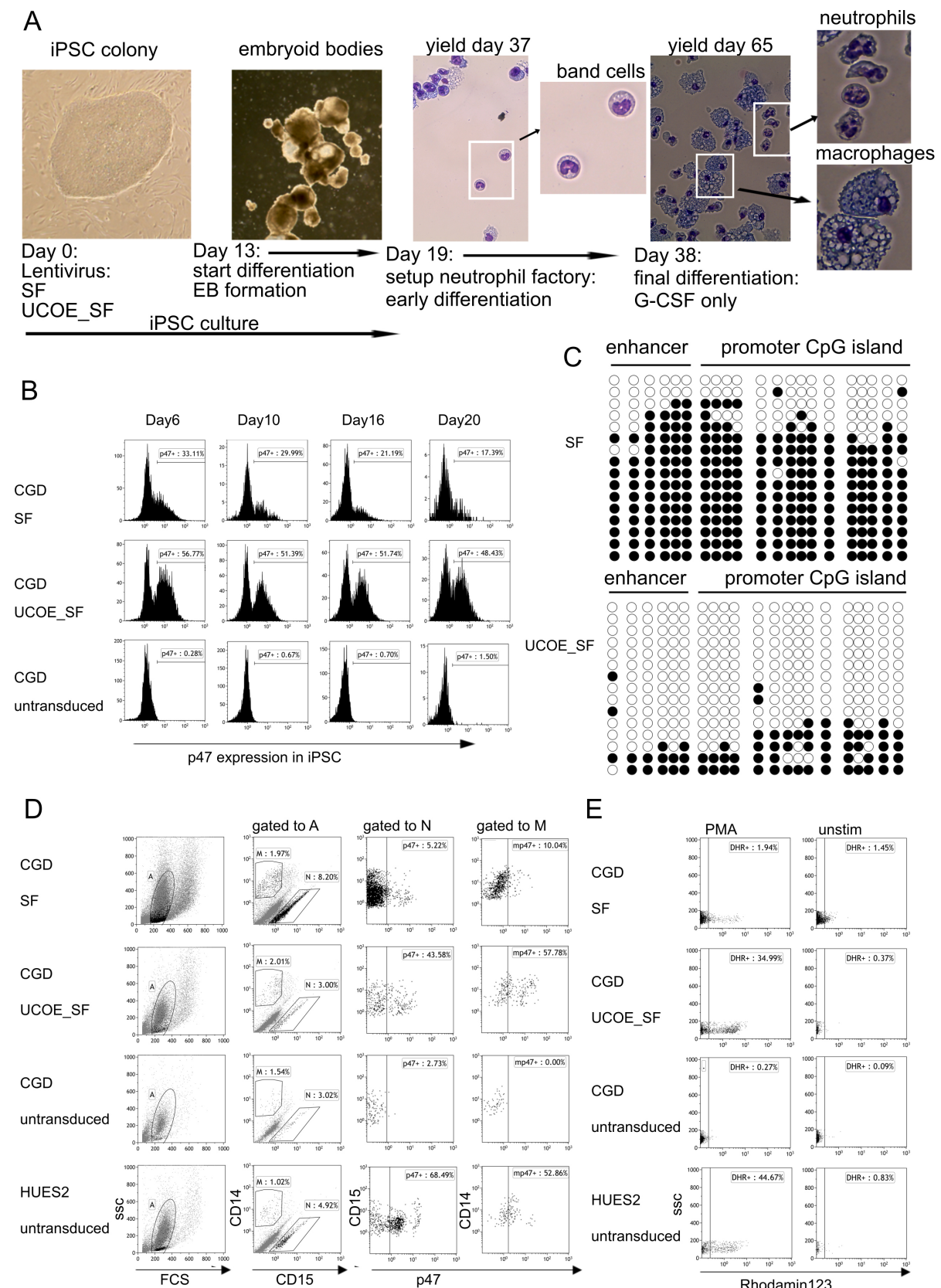
For the X-chromosomal form of CGD (X-CGD) a clinical phase I/II trial was initiated in 2005 utilizing a  $\gamma$ -retroviral GT vector. This GT vector drives gp91phox transgene expression by the viral *long terminal repeat* (LTR). The clinical trial was temporarily successful as gene modified stem cells engrafted in the bone marrow in significant amount which led to the restoration of ROS production in peripheral phagocytes and to the disappearance of life threatening infections within about 50 days after GT. Unfortunately GT was accompanied by two major adverse events; Oncogene transactivation by enhancer sequences within the LTR sequence of the GT vector led to clonal dominance which partially progressed to myelodysplastic syndrome and monosomy 7 requiring medical intervention. Furthermore an increasing discrepancy between gene marking

and functionality over time was observed resulting in decreased therapeutic efficiency. A detailed analysis revealed DNA-methylation within the viral LTR promoter sequence as the molecular cause for decreasing therapeutic efficiency [2] [3] [4].

This study focuses on the development of a silencing resistant GT vector for the p47phox deficient form of CGD. We used the constitutively active viral LTR of spleen focus forming virus (SFFV) driving p47phox transgene expression as this sequence within the LTR was sensitive to DNA methylation in the above mentioned clinical trial. To prevent transgene silencing we used the *ubiquitous chromatin opening element* (UCOE), which is published, to prevent methylation when inserted 5' of SFFV promoter [5] [6] [7] but which has not been analyzed in neutrophils in a CGD-specific context up to now. As the UCOE element possesses intrinsic promoter activity we generated various UCOE deletion constructs. Our constructs were initially tested in P19 cells known for their high DNA methylation activity. Main experiments were conducted in a p47phox CGD patient derived induced pluripotent stem cell (iPSC) line [8] as a CGD disease model to test the silencing of the GT vectors. The p47phox deficient iPSC were transduced with the GT vectors and propagated to neutrophils. The gene corrected cells were again tested for promoter methylation in iPSC, and after differentiation to neutrophils, cells were tested for expression of p47phox transgene and restoration of ROS production.

### Objective

We analyzed a UCOE construct for its capacity to protect the strongly silencing sensitive SFFV LTR promoter from inactivation in a disease model which reflects the CGD disease as well as the long term epigenetic processes observed in vivo, in order to combine this UCOE construct in a second step with a tissue-specific promoter for CGD gene therapy vector development.



## Figure Legend

Figure 1. Gene therapy of iPSC and differentiation to neutrophils.

(A) Workflow of iPSC Work. Methylene blue/eosin staining shows that early yield of neutrophil factories contain cells with band cell morphology (neutrophil progenitors), whereas late yield of the factory contains cells with mature neutrophil morphology (with the neutrophil typical multi-lobal segmented nuclei) and also macrophage morphology (with single round nuclei).

(B) p47 expression in OCT3/4 gated iPSC over time. P47 expression of UCOE\_SF\_p47 is stable over time, but decreases in SF\_p47 transduced cells.

(C) DNA methylation analysis in iPSC after 20 days of culture. CpG dinucleotides in individual subcloned PCR products are shown in horizontal orientation with black/white circles indicating methylated/unmethylated CpGs. UCOE protects the SFFV promoter from methylation, whereas unprotected SFFV promoter is strongly methylated in iPSC 20 days after transfection.

(D) Gating strategy and p47 expression in yield of neutrophil factory day 68 after transduction. p47 expression of UCOE\_SF\_p47 is maintained at high level in neutrophils (FCSlow (gate A), CD15+CD14- (gate N)) and in macrophages (FCSlow, CD14+CD15- (gate M)), but significantly reduced in SF\_p47 transduced cells in comparison to p47phox expression in iPSC on day 6 (Fig. 1B).

(E) Detection of ROS production in differentiated CD15+ neutrophils in a dihydrorhodamine 123 assay. ROS production leads to increased fluorescence resulting in a shift to the right. Percentage of ROS producing neutrophils is high for UCOE\_SF\_p47, but marginal in SF\_p47 in comparison to p47phox expression in iPSC on day 6 (Fig. 1B).

## Results & Discussion

### UCOE prevents promoter methylation in P19 cells

A previously described plasmid with full length UCOE sequence [6] was used to generate deletion constructs of this UCOE element. These constructs were then cloned into gamma-retroviral vectors 5' to the silencing sensitive SFFV promoter (Suppl. Fig. 1A). P19 cells with high DNA methylation activity were retrovirally transduced with the generated UCOE/SFFV fusion constructs and SFFV promoter methylation was analyzed (Suppl. Fig. 1B). This screen revealed two UCOE constructs which in only one orientation UCOE1794fwd and UCOE1662fwd protected the adjacent SFFV promoter from silencing. The analysis was repeated after introducing the two UCOE/SFFV fusion constructs into lentiviral vector backbones [9]. Similarly, both UCOE constructs protected the adjacent SFFV promoter from inactivation in P19 cells (Suppl. Fig. 2).

### UCOE prevents SFFV promoter methylation in iPSC and maintains stable expression in iPSC derived neutrophils

As P19 cells can't be propagated to phagocytes, we transduced cells of a p47phox-deficient iPSC cell line with the lentiviral GT vectors SF and UCOE\_SF with targeted MOI 2 and expanded them before differentiation to neutrophils alongside the untransduced CGD iPSC line as negative control and the embryonic stem cell line HUES2 as positive control. Some of the iPSC were expanded until day 20 for p47 expression analysis and promoter methylation analysis. The percentage of p47 expressing iPSC was assayed by flow cytometry at 4 timepoints. The Oct3/4 pluripotency marker was used to identify iPSC (Fig. 1B). The percentage of p47-positive cells for the SF vector transduced cells decreased from 33.11% at day 6 to 17.39% at day 20, whereas in UCOE\_SF transduced cells the percentage only marginally declined from 56.77% at day 6 to 48.43% at day 20. Methylation of the SFFV promoter was assayed at day 20 after transduction in iPSC. Similarly to P19 cells, the SFFV promoter was protected from methylation in UCOE\_SF (12.1% of CpGs methylated), whereas the SF was heavily methylated (74.1% of CpGs methylated).

### UCOE maintains stable expression and enables stable ROS production in iPSC-derived neutrophils

The iPSC were differentiated to neutrophils with an embryoid body (EB) based differentiation protocol that releases a mixture of neutrophils and macrophages into the supernatant (Fig. 1A). In early phase of culture maturation, EB supernatant contained band cells (progenitors of neutrophils). In advanced stage of culture maturation (Fig. 1A, 1D) the EB supernatant contained macrophages, and mature neutrophils with multi-lobal segmented nuclei.



p47phox transgene expression was analyzed in macrophage and neutrophil cell populations defined by surface markers (Fig. 1D). Within the CD14<sup>+</sup>/CD15<sup>-</sup> (Fig. 1D, gate M) and in the CD14<sup>-</sup>/CD15<sup>+</sup> (Fig. 1D, gate N) populations the percentages of p47 expressing cells were high for UCOE\_SF vector (gate M: 57.78%; gate N: 43.58%) and very low for unprotected SF vector (gate M: 10.04%; gate N: 5.22%). Together with the known anti-silencing properties of our UCOE construct (Fig. 1C), the low p47phox expression in the absence of the UCOE element is likely due to promoter methylation during the differentiation of iPSC to neutrophils. More important than transgene expression is functional correction of neutrophilic ROS production. The flow cytometry based dihydrorhodamine 123 (DHR) assay was used to detect ROS production upon stimulation with PMA. In CD14<sup>-</sup>/CD15<sup>+</sup> neutrophils UCOE\_SF restored ROS production in 34.99% of cells. The percentage of ROS producing neutrophils derived from UCOE\_SF transduced iPSC was in the same range as in neutrophils derived from HUES2 positive control. In contrast to these results, p47phox expression and ROS production in cells obtained from SF vector transduced iPSC was marginal (1.94%) again pointing towards epigenetic inactivation of the unprotected SFFV promoter in the SF vector.

In order to prevent silencing of the SFFV promoter we analyzed SFFV promoter and *ubiquitous chromatin opening element* (UCOE) – SFFV promoter fusion constructs for DNA methylation in a highly DNA methylating embryonic carcinoma cell line and in iPSC. The UCOE element consists of two divergently oriented CpG island housekeeping gene promoters, CBX3 and HNRPA2B1. The UCOE element was reported to be resistant to silencing and to reduce integration site effects on transgene expression levels independent of vector copy number [6]. Its anti-silencing effect was not restricted to the UCOE sequence itself but expanded to adjacent promoter sequences including an adjacent SFFV promoter [5].

This reported UCOE effect was orientation dependent. Only a UCOE element in which SFFV was fused to the CBX3 promoter element (A2B1-CBX3-SFFV termed 3'UCOE or “forward” in [5] corresponding to our reverse orientation) was reported to prevent SFFV promoter silencing [5]. In our hands, only two of our UCOE deletion constructs (1797bp (UCOE1797fwd) and 1662bp (UCOE1662fwd)) and both only in CBX3-A2B1-SFFV (forward, fwd) orientation (Suppl. Fig. 1) were able to prevent SFFV promoter silencing. It is worth noting that the UCOE elements used in our study and in the study of Zhang and coworkers differed in size.

The anti-silencing effect of our UCOE1662fwd element was independent of viral background (gamma-retrovirus and lentivirus) and could be reproduced in P19 cells and in the iPSC model. Furthermore, the GT vector Lenti UCOE 1662fwd SFFV p47 (UCOE SF) showed stable transgene expression in iPSC and in iPSC derived macrophages and neutrophils.

For the endogenous native UCOE sequence, it is known that the open DNA conformation is associated with depletion of nucleosomes and changes in histone code and demethylation of DNA around the UCOE promoters [10]. Authors could show an accumulation of histones related to active transcription of H3K9Ac, H3K14Ac, H3K4me2, H3K4me3 and histones related to open chromatin conformation H4K4Ac, H4K7ac, H4K11Ac and H4K14Ac which is mandatory for transcription. We therefore hypothesize that this histone code spreads from the UCOE element into adjacent SFFV promoter region and thereby prevents promoter methylation and transgene silencing. Recently the site with anti-silencing activity could be restricted to a core region of UCOE spanning only 455bp of the CBX3 part of UCOE in the authors 3' orientation [11], corresponding to our reverse orientation. The reason why individual constructs of different sizes possess anti-silencing activity in only one orientation remains enigmatic and requires further investigation.

## Conclusions

We have described a silencing resistant gene therapy vector with stable expression of p47phox transgene in a highly DNA methylating embryonic carcinoma cell line as well as in iPSC, and upon iPSC differentiation to neutrophils and macrophages. Due to the stable transgene expression this vector was able to facilitate full restoration of ROS production in neutrophils obtained from transduced p47phox CGD iPSC.

## Limitations

The SFFV promoter served as a model system due to its susceptibility to silencing in vivo. For gene therapy vector DNA methylation studies, the unprotected SFFV promoter and the UCOE protected SFFV promoter are reliable positive and negative controls. Further studies are required to confirm whether UCOE elements described in this study and in the study described by [11] are able to protect tissue specific promoters [9] [12] [13] as well, which might add to long term performance of these gene therapy vectors in vivo, respectively if those promoters need silencing protection after all.

## Additional Information

### Methods and Supplementary Material

Please see <https://sciencematters.io/articles/201805000005>.

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### Ethics Statement

The work with embryonic stem cell line HUES2 [14] was reviewed and approved by the U.K. Stem Cell Bank Steering Committee.

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